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Inventor: Michael Simons & Youhe Gao

For: "METHOD FOR PR-39 PEPTIDE REGULATED STIMULATION OF ANGIOGENESIS

Enclosed are:

6 sheets of drawing.

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Applicant or Patentee: Michael Simons & Youhe Gao Attorney's
Serial or Patent No.: _____ Docket No. BIS-043/ci
Filed or Issued:
For: "METHOD FOR PR-39 PEPTIDE REGULATED STIMULATION OF ANGIOGENESIS"

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Michael Simons **** Youhe Gao
NAME OF INVENTOR NAME OF INVENTOR NAME OF INVENTOR

Signature of Inventor Signature of Inventor Signature of Inventor

Date Date Date

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7 APPLICATION FOR LETTRES PATENT

8
9 BE IT KNOWN that Michael Simons and Youhe Gao have made a new and useful
10 improvement entitled "METHOD FOR PR-39 PEPTIDE REGULATED STIMULATION
11 OF ANGIOGENESIS."
12
13

RESEARCH SUPPORT

The research effort for the invention was supported in part by grants from the National Institutes of Health, grants RO1 HL 53793 and P50 HL 56993 (MS), F32 HL 10013 (RV); and by a grant from Chiron Corporation. The government has certain rights in the invention.

CROSS REFERENCE

The present application is a Continuation-In-Part of prior pending United States Patent Application Serial No. 09/276,868 filed March 26, 1999.

FIELD OF THE INVENTION

The present invention is concerned generally with the induction of angiogenesis within viable cells comprising living tissues and organs; and is particularly directed to mechanisms regulated by PR-39 peptides which result in a stimulation of angiogenesis on-demand and may be used as a controlled therapeutic treatment.

BACKGROUND OF THE INVENTION

Angiogenesis, by definition, is the formation of new capillaries and blood vessels within living tissues; and is a complex process first recognized in studies of wound

healing and then within investigations of experimental tumors. Angiogenesis is thus a dynamic process which involves extracellular matrix remodeling, endothelial cell migration and proliferation, and functional maturation of endothelial cells into mature blood vessels [Brier, G. and K. Alitalo, Trends Cell Biology 6: 454-456 (1996)]. Clearly, in normal living subjects, the process of angiogenesis is a normal host response to injury; and as such, is an integral part of the host body's homeostatic mechanisms.

It will be noted and appreciated, however, that whereas angiogenesis represents an important component part of tissue response to ischemia, or tissue wounding, or tumor-initiated neovascularization, relatively little new blood vessel formation or growth takes place in most living tissues and organs of mature adults (such as the myocardium of the living heart) [Folkman, J. and Y. Shing, J. Biol. Chem. 267: 10931-10934 (1992); Folkman, J., Nat. Med. 1: 27-31 (1995); Ware, J.A. and M. Simons, Nature Med. 3: 158-164 (1997)]. Moreover, although regulation of an angiogenic response in-vivo is a critical part of normal and pathological homeostasis, relatively little is presently known about the control mechanisms for this process.

Overall, a number of different proteins, growth factors and growth factor receptors have been found to be involved in the process of stimulation and maintenance of angiogenic responses. For example, a number of cell membrane-associated proteins are thought to be involved in the processes of angiogenesis. Such proteins include SPARC [Sage et al., J. Cell Biol. 109: 341-356 (1989); Motamed K. and E.H. Sage, Kidney Int. 51: 1383-1387 (1997)]; thrombospondin 1 and 2 respectively [Folkman, J., Nat. Med. 1: 27-31 (1995); Kyriakides et al., J. Cell Biol. 140: 419-430 (1998)]; and integrins $\alpha v\beta 5$ and $\alpha v\beta 3$ [Brooks et al., Science 264: 569-571 (1994); Friedlander et al., Science 270:

1 1500-1502 (1995)]. In addition, a major role is played by heparin-binding growth factors
2 such as basic fibroblast growth factor (bFGF) and vascular endothelial growth factor
3 (VEGF); and thus the regulation of angiogenesis is believed today to involve matrix
4 components such as extracellular heparin sulfate and core proteins such as syndecans
5 which are found at the surface of endothelial cells.

6 However, while a number of heparin binding growth factors (including VEGF,
7 FGF1 and FGF2) have been shown to promote angiogenesis in-vitro and in-vivo, their
8 process involvement appears limited to tissues demonstrating some form of inflammatory
9 response to trauma (as defined by the presence of blood-derived macrophages), be it a
10 direct tissue injury (such as wounding) or ischemia. Moreover, the presence of blood-
11 derived macrophages is also routinely associated with localized secretion of a number of
12 proteins including cytokines such as IL-2 and TNF- α , growth factors such as VEGF and
13 FGF-2, matrix metalloproteinases as well as many other biologically active molecules.
14 Accordingly, although there have been many investigations, publications, and
15 developments of these entities, there remains a general ignorance and failure of
16 understanding by research investigators and clinicians alike regarding useful and effective
17 specific means and methods for inducing angiogenesis on-demand within living cells,
18 tissues, and organs. Thus, while the value and desirability of initiating new
19 vascularization - especially using cells in localized areas on an as needed basis as well as
20 a therapeutic treatment for individual patients - is well recognized, these aims remain a
21 long sought goal yet to be achieved in a practical manner.

SUMMARY OF THE INVENTION

The present invention has multiple aspects and uses. A first aspect provides a method for stimulating angiogenesis within a targeted collection of viable cells in-situ, said method comprising the steps of:

identifying a collection of cells comprising viable cells in-situ as a target for stimulation of angiogenesis;

providing means for effecting an introduction of at least one member selected from the group consisting of the PR-39 oligopeptide collective to the cytoplasm of said targeted collection of cells;

introducing at least one member of the PR-39 oligopeptide collective to the cytoplasm of said targeted collection of cells using said effecting means;

allowing said introduced PR-39 oligopeptide collective member to interact with such proteasomes as are present within the cytoplasm of said targeted collection of cells whereby

(a) at least some of the proteasomes interact with said PR-39 oligopeptide collective member, and

(b) at least a part of the proteolytic activity mediated by said interacting proteasomes becomes selectively altered, and

(c) the selectively altered proteolytic activity of said interacting proteasomes results in a stimulation of angiogenesis in-situ within the targeted collection of viable cells.

1 A second aspect of the invention provides a method for selective inhibition of
2 proteasome-mediated degradation of peptides in-situ within a collection of viable cells,
3 said method comprising the steps of:

4 identifying a collection of cells comprising viable cells in-situ as a target;
5 providing means for effecting an introduction of at least one member selected from
6 the group consisting of the PR-39 oligopeptide collective to the cytoplasm of said targeted
7 collection of cells;

8 introducing at least one member of the PR-39 oligopeptide collective to the
9 cytoplasm of said targeted collection of cells using said effecting means;

10 allowing said introduced PR-39 oligopeptide collective member to interact with
11 such proteasomes as are present within the cytoplasm of said targeted collection of cells
12 whereby

13 (a) at least some of the proteasome interacts with the PR-39
14 oligopeptide collective member, and

15 (b) at least a part of the proteolytic activity mediated by said interacting
16 proteasomes becomes markedly altered, and

17 (c) the markedly altered proteolytic activity of said interacting
18 proteasomes results in a selective inhibition of proteasome-mediated degradation of
19 peptides in-situ within the targeted collection of cells.

1 BRIEF DESCRIPTION OF THE FIGURES

2

3 The present invention may be more fully understood and better appreciated when
4 taken in conjunction with the accompanying drawing, in which

5 Figs. 1A-1D are presentations of empirical data showing the direct interaction
6 between PR-39 peptide and the $\alpha 7$ subunit of proteasomes intracellularly;

7 Figs. 2A-2D are presentations of empirical data showing the effect of PR-39
8 peptide upon proteasome activity in-vivo;

9 Figs. 3A-3D are graphs demonstrating the results of in-vitro proteasome activity
10 assays;

11 Figs. 4A-4C are presentations of empirical data showing the in-vivo effects of PR-
12 39 peptide expression;

13 Figs. 5A-5C are photographs of representative sections showing differences in
14 vascularity among control, PR-39 peptide and FGF2 impregnated Matrigel pellets;

15 Fig. 6 is a graph providing a quantitative analysis of vascularity for the
16 representative sections of Fig. 5; and

17 Fig. 7 is a graph showing the induction of angiogenesis in-vivo using PR-39
18 peptide and short-length PR11 peptide impregnated Matrigel pellets.

19

20 DETAILED DESCRIPTION OF THE INVENTION

21

22 The present invention is a method for stimulating angiogenesis via the purposeful
23 introduction of native PR-39 peptide or a member of the PR-39 derived oligopeptide

1 family to the cytoplasm of viable cells in-situ. The PR-39 peptide or the derived member
2 of the family will interact with such proteasomes as are present intracellularly; and the
3 consequence of PR-39 peptide/proteasome interaction is the selective inactivation of
4 proteasomes such that intracellular degradation of proteins such as HIF-1 α and I κ B α is
5 diminished and a marked stimulation of angiogenesis in-situ consequently results.

6 A number of major benefits and advantages are therefore provided by the means
7 and methods comprising the present invention. These include the following:

8 1. The present invention provides an in-situ stimulation of angiogenesis. By
9 definition, therefore, both in-vivo and in-vitro circumstances of use and application are
10 envisioned and expected. Moreover, the viable cells which are the location of PR-39
11 peptide and proteasome interaction, alternatively may be isolated cells; be part of living
12 tissues comprising a variety of different cells such as endothelial cells, fibrocytes and
13 muscle cells; and may also comprise part of specific organs in the body of a living human
14 or animal subject. While the user shall choose the specific conditions and circumstances
15 for practicing the present invention, the intended scope of application and the envisioned
16 utility of the means and methods described herein apply broadly to living cells, living
17 tissues, functional organs and systems, as well as the complete living body unit as a viable
18 whole.

19 2. The present invention has a variety of different applications and uses. Of clinical
20 and medical interest and value, the present invention provides the opportunity to stimulate
21 angiogenesis in tissues and organs in a living subject which has suffered defects or has
22 undergone anoxia or infarction. A common clinical instance is the myocardial infarction
23 or chronic myocardial ischemia of heart tissue in various zones or areas of a living human

1 subject. The present invention thus provides opportunity and means for specific site
2 stimulation and inducement of angiogenesis under controlled conditions. The present
3 invention also has major research value for research investigators in furthering the quality
4 and quantity of knowledge regarding the mechanisms controlling angiogenesis under a
5 variety of different conditions and circumstances.

6 3. The present invention envisions and permits a diverse range of means for
7 introducing native PR-39 peptide or a shorter-length peptide of the oligopeptide family to
8 a specific location, site, tissue, organ, or system in the living body. A variety of different
9 routes of administration are available to the practitioner; and a wide and useful choice of
10 delivery systems are conventionally available, and in accordance with good medical
11 practice are adaptable directly for use. In this manner, not only are the means for PR-39
12 peptide introduction under the control of the user, but also the manner of localized
13 application and the mode of limiting the area of peptide introduction can be chosen and
14 controlled.

15

16 I. Underlying Direct And Indirect Mechanisms For Initiating A Stimulation
17 Of Angiogenesis

18

19 The present invention utilizes and relies upon novel and previously unknown direct
20 and indirect mechanisms of interaction between PR-39 peptide (or its shorter-length
21 homologs) and proteasomes in-situ as the basis for stimulation of angiogenesis in cells,
22 living tissues, and organs. Evidence of such multiple intracellular interactions is provided
23 by the experiments and empirical data described hereinafter. Such interactions between

1 proteasomes (and its α 7 subunit in particular) and the PR-39 peptides collectively (of any
2 size) are previously unknown; in fact, no meaningful relationship or interaction between
3 any peptide whatsoever and intracellular proteasome function has ever been proposed or
4 envisioned before the present invention was conceived or demonstrated empirically.

5 As shown experimentally hereinafter, the PR-39 peptide (and the shorter-length
6 PR-39 derived oligopeptide family members) when introduced into the cytoplasm of viable
7 cells will interact, directly and indirectly, with proteasomes. In some instances, the
8 interaction between the collective of PR-39 oligopeptides and the proteasome is direct and
9 a direct binding with the α 7 subunit of the proteasome occurs. In these instances, no
10 intermediaries or cofactors are involved in the binding reaction; and such direct α 7
11 subunit binding interactions result in a selective inactivation and inhibition of proteasome
12 function intracellularly such that expression of certain proteins such as HIF-1 α is
13 increased and stimulation of angiogenesis subsequently occurs.

14 In addition, however, alternative mechanisms of interaction in addition to and other
15 than direct inactivation of a proteasome subunit are deemed to exist and be in effect in-
16 situ for members of the PR-39 peptide collective (including native PR-39 and its
17 substituted or homologous forms). As merely an exemplary and representative instance
18 illustrating an alternative mechanism of action, the experiments and empirical data
19 presented hereinafter demonstrate that: the PR-39 peptide acts as a selective inhibitor of
20 tumor necrosis factor-a induced I κ B α degradation by proteasomes; and that PR-39 peptide
21 inhibition of I κ B α degradation is rapidly reversible (unlike the action of known inhibitory
22 compounds such as lactacysin); and that even in the presence of PR-39 peptide, the
23 intracellular expression of other proteasome-regulated proteins such as p105 and p50

1 NF κ B was unchanged. These empirical findings are meaningful evidence of, harmonious
2 with, and more consistent as demonstrating indirect mechanisms of interaction (rather than
3 any direct action effect). Accordingly, the selective and reversible nature of PR-39
4 peptide activity with regard to inhibiting I κ B α degradation is perceived to be due to a
5 prevention of recognition of ubiquitinated I κ B α by the proteasome and/or by a physical
6 blockage of its entry into the interior of the proteasome in-situ. These indirect
7 mechanisms of action are functional alternatives available on-demand and effective
8 concurrently in addition to any direct action inactivation mechanisms.

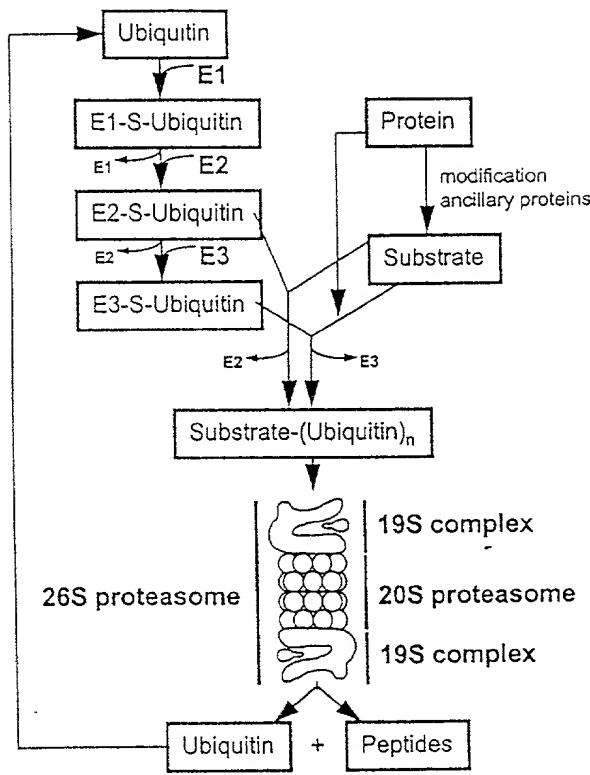
9 To obtain a demonstrable proteasome interaction and effect in-situ, the introduction
10 of native PR-39 peptide (or one of its substituted forms or its shorter-length homologs) is
11 a necessary prerequisite; and the presence of sufficient PR-39 peptide (or its substituted or
12 homologic equivalent) quantitatively to interact selectively with proteasomes intracellularly
13 within viable cells can be achieved under both in-vivo conditions and in-vitro experimental
14 circumstances.

15 The methodology and means provided by the present invention for selectively
16 inhibiting proteolysis and stimulating angiogenesis within viable cells is therefore directed
17 at and focused upon the intracellular degradation capability and functional activity of
18 proteasomes. Such selective inhibition and/or disruption of proteasome-mediated
19 degradation is achieved via the introduction of native PR-39 peptide or a member of the
20 shorter-length PR-39 derived oligopeptide family in a therapeutic regimen of treatment.

II. Proteasomes

The proteasome is a component of the ubiquitin-proteasome-dependent proteolysis system. This system plays a major role in the turnover of intracellular proteins, of misfolded proteins, and in the selective degradation of key proteins. Controlled protein degradation is an important and efficient way to remove nonfunctional proteins and/or to regulate the activity of key proteins. Target proteins are selectively recognized by the ubiquitin system and subsequently marked by covalent linkage of multiple molecules of ubiquitin, a small conserved protein. The polyubiquitinated proteins are degraded by 26S proteasome. This complex, however, is composed of two large subcomplexes: the 20S proteasome constituting the proteolytic core and the 19S regulatory complex which confers polyubiquitin binding and energy dependence. A simplified scheme of the ubiquitin pathway is depicted by Flow Scheme A below.

Flow Scheme A



* Schematic representation of the proteasome-ubiquitin pathway. Ubiquitin is first activated by a ubiquitin-activating enzyme (UBA or E1) and passed on to a ubiquitin-conjugating protein (UBC or E2). Ubiquitin is then linked directly, or with the help of ubiquitin ligases (E3), via an isopeptide bond to a lysine residue of the substrate protein. Polyubiquitinated proteins are recognized and selectively degraded by the 26S proteasome, yielding reusable ubiquitin molecules and peptides of 5 to 15 amino acids. Conversion of a protein into a substrate for ubiquitination can in certain cases occur after posttranslational modification or association with ancillary factors. Proteins can also be recognized by an E3 ubiquitin ligase without prior modification or association.

* Reproduced from Gerards et al., *CMLS* 54: 253-262 (1998)

A substantial quantum of research has been conducted to understand the architecture, assembly, and molecular biology of the proteasome. Merely representative of scientific publications in this field are the following, the individual texts of which are expressly incorporated by reference herein: Goldberg *et al.*, *Biol. Chem.* **378**: 131-140 (1997); Tanaka, K., *Biochem. Biophys. Res. Commun.* **247**: 537-541 (1998); Baumeister *et al.*, *Cell* **92**: 367-380 (1998); Gerards *et al.*, *CMLS* **54**: 253-262 (1998); Maurizi, M.R., *Curr. Biol.* **8**: R453-R456 (1998); Rechsteiner *et al.*, *J. Biol. Chem.* **268**: 6065-6068 (1993); Gerards *et al.*, *J. Mol. Biol.* **275**: 113-121 (1998); Fenteany, G. and S. Schreiber, *J. Biol. Chem.* **273**: 8545-8548 (1998); and Oikawa *et al.*, *Biochem. Biophys. Res. Commun.* **246**: 243-248 (1998).

The 20S proteasome

The degrading component in ubiquitin-dependent protealysis is the 26S proteasome. The catalytic core of this complex is the 20S proteasome, which is highly conserved and can be found in eukaryotes, archaebacteria, and some eubacteria. In eukaryotes, the amount of proteasomes can constitute up to 1% of the cell content, depending on the average protein breakdown rates of the organ. Proteasomes are localized in the nucleus and the cytosol, sometimes colocalizing or associating with the cytoskeleton. [See for example: Hilt, W. and D.H. Wolf, *Trends Biochem. Sci.* **21**: 96-102 (1996); Ciechanover, A., *Cell* **79**: 13-21 (1994); Jentseh, S. and S. Schlenker, *Cell* **82**: 881-884 (1995); Coux *et al.*, *Annu. Rev. Biochem.* **65**: 807-847 (1996); Dahlmann *et al.*, *FEBS Lett.* **251**: 125-131 (1989); Tamura *et al.*, *Curr. Biol.* **5**: 766-774 (1995); Machiels *et al.*,

1 Eur. J. Cell Biol. **66**: 282-292 (1995); Scherrer, K. and F. Bey, Prog. Nucleic Acid Res.
2 Mol. Biol. **49**: 1-64 (1994); and Gerards et al., CMLS **54**: 253-262 (1998)].

3 The first description of a "cylinder-shaped" complex with proteasome-like features
4 dates back to the late 1960s. The plethora of names given to it subsequently is a
5 reflection of the problems that were encountered over a period of two decades in trying to
6 define its biochemical properties and cellular functions. Enzymological studies revealed
7 an array of distinct proteolytic activities and led to a consensus name, 'multicatalytic
8 proteinase'. This name, however, was soon replaced by a new one, the 'proteasome'
9 emphasizing its character as a molecular machine.

10 At about the same time, it was found that the occurrence of proteasomes was not
11 restricted to eukaryotic cells. A compositionally simpler, but structurally strikingly
12 similar proteolytic complex was found in the archaeon *Thermoplasma acidophilum*, which
13 later took a pivotal role in elucidating the structure and enzymatic mechanism of the
14 proteasome.

15
16 Nomenclature

17 The 20S proteasome was independently discovered by groups working in different
18 fields, and hence was given a variety of different names. In 1970, Scherrer and
19 colleagues observed ring-shaped particles in ribosome-free messenger RNA (mRNA)
20 preparations [Sporh et al., Eur. J. Biochem. **17**: 296-318 (1970)]. Subsequently, in 1979,
21 DeMartino and Goldberg isolated a 700-kDa 'neutral protease' from rat liver [DeMartino,
22 G.N. and A.L. Goldberg, J. Biol. Chem. **254**: 3712-3715 (1997)]. Then, in 1980 Wilk
23 and Orlowski isolated a large protease complex from the pituitary that possessed three

different catalytic activities. They called it multicatalytic protease [Wilk, S. and M. Orlowski, *J. Neurochem.* **35**: 1172-1182 (1980); Wilk, S. and M. Orlowski, *J. Neurochem.* **40**: 842-849 (1983)]. Later, Monaco and McDevitt immunoprecipitated complexes consisting of low molecular weight proteins (LMPs) with a possible role in antigen presentation [Monaco, J.J. and H.O. McDevitt, *Nature* **309**: 797-799 (1984)]. Also, in 1984 this particle was called prosome, referring to its presumed role in programming mRNA translation [Schmid *et al.*, *EMBO* **3**: 29-34 (1984)]. Altogether, this complex has been given 21 different names in the literature. Since all particles were shown to be identical the name 'proteasome' (which is now generally accepted) was proposed first, referring to its proteolytic and particulate nature [Arrigo *et al.*, *Nature* **331**: 192-194 (1988); Faulkenburg *et al.*, *Nature* **331**: 190-192 (1988); Brown *et al.*, *Nature* **353**: 355-357 (1991)].

Overall characteristics and properties

The 20S proteasome is the major cytosolic protease in eukaryotic cells and is the proteolytic component of the ubiquitin-dependent degradative pathway. Proteasomes are also found in some, but not all, archaebacteria and eubacteria, and in eukaryotes. True proteasomes are composed of 28 subunits, 14 each of two different classes - non-catalytic alpha (α) and catalytically-active beta (β) subunits. The subunits are arranged in rings of seven subunits, all of a single type. The 20S proteasome is a stack of four rings, two inner beta rings flanked by the alpha rings. The junction between the beta rings produces a remarkable structural feature of proteasomes - an interior aqueous cavity large enough to accommodate about 70 kDa of protein and accessible only through narrow axial

1 channels in the rings. The catalytic sites are located on the beta subunits within the
2 aqueous cavity. Isolation of the catalytic sites in this way, and the limited access via
3 narrow channels, serves to compartmentalize proteolysis, allowing degradation of only
4 those proteins that can be actively translocated into the interior of the proteasome.

5

6 Structure and subunit components

7 The 20S proteasome has a cylindrical or barrel-like structure, typically 14.8 nm in
8 length and 11.3 nm in diameter. It is composed of 28 subunits and arranged in four
9 stacked rings, resulting in a molecular mass of about 700 kDa. This overall structural
10 architecture is conserved from bacteria to man.

11 In eukaryotes, including humans, 14 different subunits, ranging from 21 kDa to 32
12 kDa, are present in the complex. Based on the sequence homology with the *T.*
13 *acidophilum* α - or β -subunit, the eukaryotic subunits are divided into α -type and β -type,
14 respectively [Zwicki et al., *Biochemistry* 31: 964-972 (1992); Heinemeyer et al.,
15 *Biochemistry* 33: 12229-12237 (1994); Coux et al., *Mol. Gen. Genet.* 245: 769-780
16 (1994)]. Table 2 shows some characteristics and alternative names of the subunits of the
17 human and yeast 20S proteasome using the older and the new nomenclature proposed by
18 Groll and coworkers [Groll et al., *Nature* 386: 463-471 (1997)]. Immuno-electron
19 microscopy (EM) studies also revealed that the eukaryotic α -type subunits reside in the
20 outer rings and the β -type subunits in the inner rings. Furthermore, these studies
21 indicated that in the eukaryotic 20S proteasome seven different subunit constitute a ring,
22 each subunit located at a defined position [Kopp et al., *J. Mol. Biol.* 229: 14-19 (1993);
23 Kopp et al., *J. Mol. Biol.* 248: 264-272 (1995); Schauer et al., *J. Struct. Biol.* 111: 135-

1 147 (1993); Kopp et al., Proc Natl Acad Sci USA 94: 2939-2944 (1997)]. Therefore, the
2 eukaryotic proteasome assembles as an $\alpha_{1-7}\beta_{1-7}\beta_{1-7}\alpha_{1-7}$ particle. The typical human
3 structure and assembly is illustrated by Table 3.

4

Table 2: Nomenclature and molecular masses of proteasomal subunits

<u>Systematic name</u>	<u>Human gene</u>	<u>Yeast gene</u>	Molecular mass of human subunit (kDa)
α_1	HsPROS27 HsIota	C7 PRS2	27.4
α_2	HsC3	Y7	25.9
α_3	HsC9	Y13	29.5
α_4	XAPC7 HsC6	PRE6	27.9
α_5	HsZeta	PUP2	26.4
α_6	HsPROS30 HsC2	PRE5	30.2
α_7	HsC8	C1 PRS1	28.4
β_1	HsDelta Y	PRE3	25.3 (21.9)
β_{1i}	LMP2		23.2 (20.9)
β_2	Z	PUP1	30.0 (24.5)
β_{2i}	MECL1		28.9 (23.8)
β_3	HsC10-11	PUP3	22.9
β_4	HsC7-1	PRE1 C11	22.8
β_5	MB1 X	PRE2	nd (22.4)
β_{5i}	LMP7		30.4 (21.2)
β_6	HsC5	C5 PRS3	26.5 (23.3)
β_7	HsBPROS26 HsN3	Pre4	29.2 (24.4)

* Reproduced from Gerards et al., CMLS 54: 253-262 (1998)

Table 3: Schematic representation of the human 20S proteasome*



* Reproduced from Gerards et al., CMLS 54: 253-262 (1998)

1 Proteolytic activity

2 The first report on the multicatalytic properties of the proteasome stems from
3 1983, when three different proteolytic activities were distinguished: 'trypsin-like',
4 'chymotrypsin-like' and 'peptidylglutamyl-peptide hydrolase' activity [Wilk, S. and M.
5 Orlowski, J. Neurochem. **40**: 842-849 (1983)]. These three proteasomal activities refer to
6 peptide bond cleavage at the carboxyl side of basic, hydrophobic and acidic amino acid
7 residues, respectively. They were identified using short synthetic peptide substrates and
8 are believed to be catalyzed at independent sites - in part because the different proteolytic
9 activities respond differentially to various activators and inhibitors. With similar
10 approaches, at least two additional proteolytic activities have been recently described
11 [Orlowski et al., Biochemistry **32**: 1563-1572 (1993); Orlowski, M., Biochemistry **29**:
12 10289-10297 (1990); Rivett, A.J., Biochem. J. **291**: 1-10 (1993)].

13

14 The Progressive Degradation Of Protein Substrates

15 Recent studies have also revealed a fundamental new property of the proteasome
16 that clearly distinguishes it from conventional proteases: i.e., this particle degrades a
17 protein substrate all the way to small peptides, before attacking another protein substrate
18 [Akopian et al., J. Biol. Chem. **272**: 1791-1798 (1997)]. Because the proteasome's
19 multiple active sites are located in its central chamber and because diffusion of a peptide
20 substrate into this compartment must be a slow process, these particles function in a
21 highly processive fashion; i.e., they have mechanisms of action to bind tightly protein
22 substrates and to make multiple cleavages in the polypeptide before releasing the peptide
23 products. Moreover, the ratio of new peptides generated to the number of substrate

1 molecules consumed is constant during the reaction. In other words, as peptides
2 accumulated, they were not hydrolyzed further, even during prolonged incubations, where
3 up to half of the substrate molecules were consumed. Equally important, the
4 disappearance of these substrate molecules coincided exactly with the appearance of small
5 peptide products [Goldberg *et al.*, *Biol. Chem.* **378**: 131-140 (1997)]. These
6 observations, together with the finding that the pattern of the products is independent of
7 time, established that processive degradation is a general feature of the 20S proteasome
8 [Gerard *et al.*, *CMLS* **54**: 253-262 (1998)].

9 The contribution of each individual active center and proteolytic activity to the
10 degradation of longer peptides and complete proteins is presently unknown. Nevertheless,
11 proteasomes are able to cleave behind most amino acids in a protein. Thus, the 20S
12 proteasome is in fact a nonspecific endopeptidase. In addition, however, the generated
13 (degraded) peptides fall into a rather narrow size range of 6 to 10 amino acids in length,
14 demonstrating the existence of a kind of 'molecular ruler'. The average length of the
15 degradation products is typically 7 to 8 amino acids; this finding is in agreement with the
16 distance between the active sites in the proteasome. Similar nonspecific endopeptidase
17 activity and size distribution of degradation products from whole proteins was observed for
18 proteasomes generally and by proteasomes of human origin in particular.

19 Other features of the 20S proteasome degradation are also unique. While unfolded
20 peptides are usually digested, most native proteins are resistant to proteolytic degradation
21 by the 20S proteasome *in vitro*. However, denaturation of the substrate protein by
22 oxidation or reduction of disulphide bridges can render it accessible to degradation by
23 proteasomes. Also, small gold particles with a diameter of 2 nm containing unfolded

1 substrate cannot enter the proteasome. These characteristics show that a relatively narrow
2 opening controls access to the inner proteolytic compartment of the proteasome.

3

4 III. The PR-39 Oligopeptide Collective

5

6 Native PR-39 peptide is a substance belonging to the cathelin family of proteins;
7 the mature peptide is 39 amino acids in length in the naturally occurring state; and the
8 peptide is able to exert a variety of activities and cause different cellular outcomes.

9 Although first identified as a membrane permeating antibacterial peptide found in the
10 intestine of pigs [Agerberth *et al.*, Eur. J. Biochem. **202**: 849-854 (1991)], this peptide
11 was subsequently isolated from wounds where it could simultaneously reduce infection and
12 influence the action of growth factors, matrix components, and other cellular effectors
13 involved in wound repair [Gallo *et al.*, Proc. Natl. Acad. Sci. USA **91**: 11035-11039
14 (1994); Gallo *et al.*, J. Invest. Dermatol. **104**: 555 (1995)]. The structure and membrane
15 interactions of native PR-39 peptide have also been elucidated [Cariaux *et al.*, Eur. J.
16 Biochem. **224**: 1019-1027 (1994)] and the complete amino acid sequences of native PR-39
17 peptide and its various substituted forms have been reported [PCT Publication No. WO
18 92/22578 published 23 December 1992].

19 More recently, the native PR-39 peptide was shown to possess a syndecan-inducing
20 activity in furtherance of its wound healing capabilities; and while renamed a "synducin",
21 was shown to induce cellular production of two specific proteoglycans, syndecan-1 and
22 syndecan-4, within living mesenchymal cells [U.S. Patent No. 5,654,273]. Overall,
23 native PR-39 peptide has been shown to play a role in several inflammatory events

1 including wound healing and myocardial infarction [Gallo *et al.*, Proc. Natl. Acad. Sci.
2 USA 91: 11035-11039 (1994); Li *et al.*, Circ. Res. 81: 785-796 (1997)]; and the native
3 peptide has been shown to be taken up rapidly by a number of different cell types
4 including meschymal cells and endothelial cells [Chan, Y.R. and R.L. Gallo, J. Biol.
5 Chem. 273: 28978-28985 (1998)].

6

7 The PR-39 peptide grouping

8 Native PR-39 peptide is composed of the 39 amino acid sequence shown below
9 (and also by Table 4).

10

11 PR-39: Arg-Arg-Arg-Pro-Arg-Pro-Pro-Tyr-Leu-Pro-Arg-Pro-Arg-Pro-Pro-Phe-
12 Phe-Pro-Pro-Arg-Leu-Pro-Pro-Arg-Ile-Pro-Pro-Gly-Phe-Pro-Pro-Arg-Phe-
13 Pro-Pro-Arg-Phe-Pro

14

15 As conventionally known and reported [see for example, U.S. Patent No.
16 5,654,273], the specific peptide can be substituted using conservative substitutions of
17 amino acids having the same or functionally equivalent charge and structure, except for
18 the required amino acid sequence "Arg-Arg-Arg" at the N-terminus and the intermediate
19 amino acid sequences "Pro-Pro-X-X-Pro-Pro-X-X-Pro" and "Pro-Pro-X-X-X-Pro-Pro-X-
20 X-Pro" where X can be substituted freely using any amino acid. Thus, all of the
21 preferred substituted amino acid sequences are of about the same size and each differ
22 from the native PR-39 peptide sequence only by substitutions in the intermediate portions
23 of the structure.

1 The PR-39 derived oligopeptide family

2 In addition to the conventionally known native PR-39 peptide amino acid residue
3 sequence and its readily recognizable substituted forms as described above, an entirely
4 novel and unforeseen family of PR-39 derived oligopeptide structures is provided by the
5 present invention for use. This previously unknown family of PR-39 derived
6 oligopeptides is constituted of members which individually will cause a selective inhibition
7 of proteasome-mediated degradation of peptides in-situ after introduction intracellularly to
8 a viable cell.

9 Each member of this PR-39 derived oligopeptide family presents characteristics
10 and properties which are commonly shared among the entire membership. These include
11 the following:

- 12 (i) each peptide sequence is less than 39 amino acid residues in length in every
13 embodiment, and preferably is less than 20 residues in size in the best mode;
- 14 (ii) each short-length peptide sequence is at least partially homologous (or
15 analogous) with the N-terminal amino acid residues of the native PR-39 peptide, and
16 preferably is completely identical or markedly similar to the N-terminal end residues of
17 the native PR-39 peptide;
- 18 (iii) each short-length peptide is able to interact in-situ with at least the $\alpha 7$
19 subunit of such proteasomes as are present within the cytoplasm of the cell; and
- 20 (iv) each short-length peptide sequence is able to alter markedly the proteolytic
21 activity of proteasomes with an interacting $\alpha 7$ subunit such that a selective increased
22 expression of specific proteins (such as $I\kappa B\alpha$ and $HIF-1\alpha$) occurs in-situ.

1 Merely as illustrative examples and preferred embodiments of the broad
2 membership constituting this PR-39 derived oligopeptide family, the members comprising
3 15, 11 and 8 amino acid residues respectively in length are presented below as the PR15,
4 PR11, and PR8 entities respectively. For comparison purposes only, the complete amino
5 acid sequence of the native PR-39 peptide is presented as well.

6

7 PR-39: 1 2 3 4 5 6 7 8 9 10 11 12 13
8 Arg-Arg-Arg-Pro-Arg-Pro-Pro-Tyr-Leu-Pro-Arg-Pro-Arg-
9
10 14 15 16 17 18 19 20 21 22 23 24 25 26
11 Pro-Pro-Pro-Phe-Phe-Pro-Pro-Arg-Leu-Pro-Pro-Arg-Ile-
12
13 27 28 29 30 31 32 33 34 35 36 37 38 39
14 Pro-Pro-Gly-Phe-Pro-Pro-Arg-Phe-Pro-Pro-Arg-Phe-Pro
15
16
17

18 PR-15: 1 2 3 4 5 6 7 8 9 10 11 12 13
19 Arg-Arg-Arg-Pro-Arg-Pro-Pro-Tyr-Leu-Pro-Arg-Pro-Arg-
20
21 14 15
22 Pro-Pro
23
24
25

26 PR-11: 1 2 3 4 5 6 7 8 9 10 11
27 Arg-Arg-Arg-Pro-Arg-Pro-Pro-Tyr-Leu-Pro-Arg
28
29
30

31 PR-8: 1 2 3 4 5 6 7 8
32 Arg-Arg-Arg-Pro-Arg-Pro-Pro-Tyr
33
34

35 The PR-39 Oligopeptide Collective

36 Terminology and nomenclature often pose problems for the reader as to what
37 precisely is meant. Accordingly, for definitional purposes, avoidance of ambiguities, and
38 clarity of understanding, the following terms and titles will be employed herein. The term
39 "PR-39 peptides grouping" includes by definition the native PR-39 structure and all

1 substituted forms conventionally known of the naturally occurring 39 length amino acid
2 sequence. In distinction, the term "PR-39 derived oligopeptide family" and its members
3 includes by definition all the previously unknown shorter-length homologs and analogs of
4 the native PR-39 structure as described above. Finally, the umbrella term and category
5 title "PR-39 oligopeptide collective" includes by definition both the 'PR-39 peptide
6 grouping' as well as the 'PR-39 derived oligopeptide family' members, and identifies any
7 and all individual structures falling into either of the two subset categories.

8

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Table 4:

(1) GENERAL INFORMATION:

- (i) APPLICANT: Children's Medical Center Corporation
- (ii) TITLE OF INVENTION: Synducin Mediated Modulation of Tissue Repair
- (iii) NUMBER OF SEQUENCES: 4
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Patrea L. Pabst
 - (B) STREET: 2800 One Atlantic Center
1201 West Peachtree
 - (C) CITY: Atlanta
 - (D) STATE: Georgia
 - (E) COUNTRY: USA
 - (F) ZIP: 30309-3450
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: (404)-873-8794
 - (B) TELEFAX: (404)-815-8795

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 39 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO
- (x) PUBLICATION INFORMATION:
 - (A) AUTHORS: Lee, Jong-Youn
Boman, Hans G.
Mutt, Viktor
Jornvall, Hans
 - (B) TITLE: Novel Polypeptides And Their Use
 - (C) JOURNAL: PCT WO 92/22578
 - (G) DATE: 12/23/92
 - (K) RELEVANT RESIDUES IN SEQ ID NO:1: FROM 1 TO 39
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Arg Arg Arg Pro Arg Pro Pro Tyr Leu Pro Arg Pro Arg Pro
Pro Pro
1 5 10

15

Phe Phe Pro Pro Arg Leu Pro Pro Arg Ile Pro Pro Gly Phe
Pro Pro
20 25 30

Arg Phe Pro Pro Arg Phe Pro
35

1 Synthesis

2 The PR-39 peptide can be synthesized using standard amino acid synthetic
3 techniques. An example is the conventionally used solid phase synthesis [Merrifield, J.,
4 J. Am. Chem. Soc. 85: 2149 (1964)] described in U.S. Patent No. 4,244,946, wherein a
5 protected alpha-amino acid is coupled to a suitable resin, to initiate synthesis of a peptide
6 starting from the C-terminus of the peptide. Other methods of peptide synthesis are
7 described in U.S. Patent Nos. 4,305,872 and 4,316,891, the teachings of which are
8 incorporated herein. These methods can be used to synthesize peptides having identity
9 with the native PR-39 peptide amino acid sequence described herein, or to construct
10 desired substitutions or additions of specific amino acids, which can be screened for
11 content and evaluated for activity. PR-39 can also be commercially obtained from
12 Magainin, Inc. (Plymouth Meeting, PA).

13

14 Pharmaceutical Formats

15 After synthesis or purchase, the PR-39 peptides (as a family of homologs and
16 analogs with substituted amino acid residues) can be introduced as a peptide-containing
17 preparation in a pharmaceutically acceptable format.

18 The PR-39 can be administered and introduced in-vivo systemically, topically, or
19 locally. The peptide can be administered as the peptide or as a pharmaceutically
20 acceptable acid- or base-addition salt, formed by reaction with an inorganic acid (such as
21 hydrochloric acid, hydrobromic acid, perchloric acid, nitric acid, thiocyanic acid, sulfuric
22 acid, and phosphoric acid); or with an organic acid (such as formic acid, acetic acid,
23 propionic acid, glycolic acid, lactic acid, pyruvic acid, oxalic acid, malonic acid, succinic

1 acid, maleic acid, and fumaric acid); or by reaction with an inorganic base (such as
2 sodium hydroxide, ammonium hydroxide, potassium hydroxide); or with an organic base
3 (such as mono-, di-, trialkyl and aryl amines and substituted ethanolamines).

4 PR-39 peptide and any of the PR-39 derived oligopeptide family members may
5 also be conjugated to sugars, lipids, other polypeptides, nucleic acids and PNA; and
6 function in-situ as a conjugate or be released locally after reaching a targeted tissue or
7 organ. The PR-39 family of peptides may also be linked to targeting compounds for
8 attachment in-situ to a specific cell type, tissue or organ.

9

10 IV. Means For Introduction Of PR-39 Peptide

11 And/Or Its Shorter-Length Derived Homologs

12

13 DNA Fragments and Expression Vectors

14 A variety of means and methods are conventionally known and presently available
15 to the user or practitioner of the present invention in order to introduce PR-39 peptide (or
16 a derived oligopeptide family member) to living cells and tissues. One desirable means
17 uses a prepared DNA sequence fragment encoding the PR-39 peptide (or a shorter-length
18 homolog) in a suitable vector as the means of introduction to the intended target in-situ.
19 These means for delivery envision and include in-vivo use circumstances; ex-vivo
20 specimens and conditions; and in-vitro cultures. In addition, the present invention intends
21 and expects that the prepared DNA sequence fragment coding for PR-39 peptide (or
22 shorter-length homologs) has been inserted in a suitable expression vector and will be used
23 in a route of administration for delivery to living tissues comprising endothelial cells, and

1 typically vascular endothelial cells which constitute the basal layer of cells within
2 capillaries and blood vessels generally. Clearly, the cell recipients themselves are thus
3 eukaryotic in origin, typically mammalian cells from human and animal sources; and most
4 typically would include the higher orders of mammals such as humans and domesticated
5 mammalian animals kept as pets or sources of food intended for future consumption.
6 Accordingly, the range of animals includes all domesticated varieties involved in nutrition
7 including cattle, sheep, pigs and the like; as well as those animals typically used as pets or
8 raised for commercial purposes including horses, dogs, cats, and other living mammals
9 typically living with and around humans.

10 Clearly, the expression vectors must be suitable for transfection of endothelial cells
11 in living tissues of mammalian origin and thus be compatible with that type and condition
12 of cells under both in-vivo and/or in-vitro conditions. The expression vectors thus
13 typically include plasmids and viruses as expression vectors.

14 Also, both the plasmid based vectors and the viral expression vectors constitute
15 conventionally known means and methods of introduction which are conventionally
16 recognized today as "gene therapy" modes of delivery. However, this overall approach is
17 not the only means and method of delivery available for the present invention.

18

19 Direct Introduction of Previously Synthesized PR-39 Peptides or a PR-39 Derived
20 Oligopeptide Family Member

21 PR-39 peptide or an oligopeptide family member can be introduced directly as a
22 synthesized compound to living cells and tissues via a range of different delivery means.
23 These include the following.

1 1. Intracoronary delivery is accomplished using catheter-based deliveries of
2 synthesized PR-39 peptide (or homolog member) suspended in a suitable buffer (such as
3 saline) which can be injected locally (i.e., by injecting into the myocardium through the
4 vessel wall) in the coronary artery using a suitable local delivery catheter such as a 10mm
5 InfusaSleeve catheter (Local Med, Palo Alto, CA) loaded over a 3.0mm x 20mm
6 angioplasty balloon, delivered over a 0.014 inch angioplasty guidewire. Delivery is
7 typically accomplished by first inflating the angioplasty balloon to 30 psi, and then
8 delivering the protein through the local delivery catheter at 80 psi over 30 seconds (this
9 can be modified to suit the delivery catheter).

10 2. Intracoronary bolus infusion of PR-39 peptide (or a short-length homolog)
11 synthesized previously can be accomplished by a manual injection of the substance
12 through an Ultrafuse-X dual lumen catheter (SciMed, Minneapolis, MN) or another
13 suitable device into proximal orifices of coronary arteries over 10 minutes.

14 3. Pericardial delivery of synthesized PR-39 peptide (or a shorter-length homolog) is
15 typically accomplished by instillation of the peptide-containing solution into the pericardial
16 sac. The pericardium is accessed via a right atrial puncture, transthoracic puncture or via
17 a direct surgical approach. Once the access is established, the peptide material is infused
18 into the pericardial cavity and the catheter is withdrawn. Alternatively, the delivery is
19 accomplished via the aid of slow-release polymers such as heparin-alginate or ethylene
20 vinyl acetate (EVAc). In both cases, once the PR-39 peptide (or homolog) is integrated
21 into the polymer, the desired amount of PR-39/polymer is inserted under the epicardial fat
22 or secured to the myocardial surface using, for example, sutures. In addition, the PR-
23 39/polymer can be positioned along the adventitial surface of coronary vessels.

1 4. Intramyocardial delivery of synthesized PR-39 peptide (or a shorter-length
2 homolog) can be accomplished either under direct vision following thoracotomy or using
3 thoracoscope or via a catheter. In either case, the peptide containing solution is injected
4 using a syringe or other suitable device directly into the myocardium. Up to 2 cc of
5 volume can be injected into any given spot and multiple locations (up to 30 injections) can
6 be done in each patient. Catheter-based injections are carried out under fluoroscopic,
7 ultrasound or Biosense NOGA guidance. In all cases after catheter introduction into the
8 left ventricle the desired area of the myocardium is injected using a catheter that allows
9 for controlled local delivery of the material.

10

11 Pharmaceutical Carriers Of PR-39 Peptides or a PR-39 Derived Oligopeptide Family

12 Member

13 A range of suitable pharmaceutical carriers and vehicles are known conventionally
14 to those skilled in the art. Thus, for parenteral administration, the compound will
15 typically be dissolved or suspended in sterile water or saline.

16 For enteral administration, the PR-39 peptide or homologous oligopeptide of
17 choice will be typically incorporated into an inert carrier in tablet, liquid, or capsular
18 form. Some suitable carriers are starches and sugars; and often include lubricants,
19 flavorings, binders, and other materials desirable in tablet making procedures.

20 The PR-39 peptide and oligopeptide family of compounds can also be administered
21 topically by application of a solution, cream, gel, or polymeric material (for example, a
22 PluronicTM, BASF).

1 As an alternative, the chosen peptide can be administered in liposomes or
2 microspheres (or microparticles), which can be injected for local or systemic delivery.
3 Methods for preparing liposomes and microspheres for administration to a patient are
4 conventionally known to those skilled in the art. For example, U.S. Patent No.
5 4,789,734 describes methods for encapsulating biological materials in liposomes.
6 Essentially, the material is dissolved in an aqueous solution, the appropriate phospholipids
7 and lipids added, along with surfactants if required, and the material dialyzed or
8 sonicated, as necessary. See also, G. Gregoriadis, Chapter 14, "Liposomes", Drug
Carriers in Biology and Medicine, chap. 14, pp. 287-341 (1979). Microspheres formed
9 of polymers or proteins are well known to those skilled in the art, and can be tailored for
10 passage through the gastrointestinal tract directly into the bloodstream. Alternatively, the
11 compound can be incorporated and the microspheres, or composite of microspheres,
12 implanted from days to months. See, for example, U.S. Patent Nos. 4,906,474;
13 4,925,673; and 3,625,214.

15

16 Exemplary Introductions And Preferred Routes of Administration

17 A variety of approaches, routes of administration, and delivery methods have been
18 identified herein and are available for introduction of PR-39 peptide and the derived
19 family of oligopeptides. It is envisioned, however, that a majority of the approaches and
20 routes of administration described herein will be medical applications and specific clinical
21 approaches intended for use with individual human patients having specified medical
22 problems and diagnosed pathologies. It is expected, accordingly, that the reader is
23 familiar generally with the typical clinical human problem, pathology, and medical

1 conditions described herein; and therefore will be able to follow and easily understand the
2 nature of the intervention clinically using the present invention and the intended outcome
3 and result of the clinical treatment - particularly as pertains to the stimulation of
4 angiogenesis under in-vivo treatment conditions. A representative listing of preferred
5 clinical approaches is given by Table 5 below.

6

CONFIDENTIAL INFORMATION

Table 5

Preferred Routes of Administration

Catheter-based (intracoronary) injections and infusions;

Direct myocardial injection
(intramyocardial guided);

Direct myocardial injection
(direct vision-epicardial-open chest or under thoroscope guidance);

Local intravascular delivery;

Liposome-based delivery;

Delivery in association with receptor-specific peptides;

Oral delivery;

In instances of peripheral vascular disease:
- intramuscular injection
- intraarterial injection and/or infusion.

V. Experiments and Empirical Data

To demonstrate the merits and value of the present invention, a series of planned experiments and empirical data are presented below. It will be expressly understood, however, that the experiments described and the results provided are merely the best evidence of the subject matter as a whole which is the invention; and that the empirical data, while limited in content, is only illustrative of the scope of the invention envisioned and claimed.

Introduction

Proteolytic degradation in mammalian cells is known to proceed via two distinct pathways: lysosome-dependent degradation and proteasome-dependent. The proteasome in its pathway plays a key role in proteolysis of intracellular proteins which are marked for degradation by the ubiquitin system. The multienzyme complex involved in these events, the 26S proteasome, consists of a 20S catalytic proteasome "core" and two 19S caps that bind ubiquitylated proteins, as has been described in detail previously herein. Proteasome-mediated proteolysis is a principal event quantitatively controlling intracellular levels of a number of different proteins including hypoxia-inducing factor (HIF)-1 α , heat shock protein HSP70, protooncogenes c-Fos, c-Jun and c-Mos, NF κ B inhibitor I κ B α , and various cyclins. In addition, the proteasome also is known to play a critical role in the specific processing and presentation of major histocompatibility complex (MHC) class I-restricted antigens as well as provide partial proteolytic cleavage of p105 NF κ B to the active p50 subunit.

1 The PR-39 peptide, belonging to the cathelin family of proteins, plays an important
2 role in several inflammatory events including wound healing and myocardial infarction.
3 The PR-39 peptide typically is rapidly taken up by a number of different cell types
4 including endothelial cells; and prolonged treatment with PR-39 peptide leads to increased
5 cell growth and angiogenesis. However, the mechanism of action for this peptide activity
6 has yet to be understood or defined. The experiments and data presented below reveal for
7 the first time the nature and detailed intracellular actions exerted by the PR-39 peptide.

8

9 **Methods and Materials:**

10

11 **Yeast two-hybrid screening**

12 Two-hybrid screening was carried out using MATCHMAKER GAL4 System 2
13 (Clontech) with exon 4 of the porcine PR-39 gene as a bait to screen the mouse embryo
14 3T3 cDNA library in yeast CG1945.

15

16 **Cell culture studies**

17 U937 cells (ATCC) grown in RPMI medium 1640 with 10% FBS (Gibco-BRL)
18 and ECV cells were treated with synthetic PR-39, lactacystin (CalBiochem, 426100) or
19 MG132 (CalBiochem, 474790) at concentration indicated in the presence of 100 mM
20 cyclohexamide 20 mM chloroquine [Merin *et al.*, *J. Biol. Chem.* **273**: 6373-6379 (1995)].
21 After 45 min. of incubation, TNF α (1 ng/ml) was added. After 5 min of 37°C
22 incubation, the cells were lysed in SDS-PAGE loading buffer. Following SDS-PAGE of
23 the total protein extract, I κ B- α and NF- κ B p105 p50 expressions were determined by

1 Western blotting with anti-human antibodies (Santa Cruz, sc-203, sc114G). For studies of
2 HIF-1 α and VEGF expression, ECV cells were cultured in a hypoxia chamber (5%
3 CO₂/95% N₂) at 37°C for 16 hr. HIF-1 α was immunoprecipitated with anti-HIF-1 α mAb
4 (OZ12 1:5) in RIPA buffer and Western blotting with anti-HIF-1 α mAb (OZ15 1:10)
5 (courtesy of Dr. A. King, DFCI, Boston). VEGF expression was shown by Western
6 blotting of hypoxia treated ECV cell lysate with anti-human VEGF antibody (Santa Cruz,
7 sc-152). For HSP70 expression, U937 was treated for 3 hr, harvest with SDS-PAGE
8 loading buffer, Western blotting with anti-human HSP70 polyclonal antibody (Santa Cruz,
9 sc-1060).

10

11 **In-vitro proteasome activity assays**

12 Rabbit muscle 20S proteasome preparation (courtesy of Dr. M. Sherman, BBRI,
13 Boston) was used for all studies. For determination of proteasome activity, 5 μ l of 1:10
14 diluted proteasome preparation was incubated at room temperature in eukaryotic
15 proteasome assay buffer (20 mM Tris-HCl pH 8.0, 0.5 mM EDTA and 0.01% SDS) with
16 20 μ M proteasome substrates (CalBiochem, 539140-3) and PR-39 or other proteasome
17 inhibitor at indicated concentration [Rock et al., Cell 78: 761-771 (1994)]. The extent of
18 substrate degradation was monitored continuously by fluorescence spectrophotometry (380
19 nm excitation, 460nm emission Hitachi F-2000) for 10 min.

Experiment 1:

This experiment was designed to reveal the ability of PR-39 peptide to affect proteasome function. To test this capability, the effect of PR-39 administration upon α_7 subunit processing was empirically determined. The results are illustrated by Figs. 1A-1D respectively.

Experimentally, a peptide corresponding to the 4th exon porcine PR-39 gene sequence was used to generate a rabbit polyclonal antibody RPE4. Full length porcine cDNA (containing leader sequence) and a sequence corresponding to the 4th exon of porcine PR-39 gene were cloned into eukaryotic expression vector pGRE5-2 (USB). These expression constructs were then used to stably transfect an immortalized human endothelial cell line (ECV304, ATCC). For co-immunoprecipitation, wild type ECV, full length PR-39 (ECV-PR39) and exon 4 PR39 and exon 4 PR39 (ECV-E4) transfected cells were cultured in Medium 199 with 10% fetal bovine serum (FBS) and penicillin/streptomycin. Cells were lysed with RIPA buffer; immunoprecipitated with 10 µg affinity purified rabbit anti-PR39 antibody; and following Protein A-Sepharose purification and SDS-PAGE, subjected to immunoblotting with 1:1000 mouse anti-HC8 mAb (Affiniti Research Products Limited UK, PW8110).

Figs. 1A-1D show the interactions of PR-39 peptide and the α 7 subunit of proteasomes. Fig. 1A recites the cDNA sequence of cloned mouse α 7 subunit (top; GeneBank accession number AF055983) and corresponding human HC8 subunit of 20S proteasome. Fig. 1B shows the sequence alignment of C-terminal tails mouse α subunits of 20S proteasome. Fig. 1C shows a deletion analysis of α 7-PR39 binding. Deletion mutants of the mouse α 7 subunit were cloned into an yeast-two hybrid vector and the

1 extent of growth of lacZ⁺ colonies on selective medium following co-transformation with
2 PR-39 construct in the yeast CG1945 was determined. It is noted that only full length α 7
3 construct was able to bind to PR-39. Finally, Fig. 1D shows the co-immunoprecipitation
4 of PR-39 and α 7 subunit in ECV cells.

It will be noted also that Fig. 1 represents the evidence of four clones growing on selective media and demonstrating lacZ staining. All four clones encoded overlapping identical cDNA sequences highly homologous to the human sequence of α 7 (HC8) subunit of proteasome (Fig. 1A). Similar to all α subunits of the 20S proteasome, the cloned mouse protein possesses a highly conserved N-terminal region; in addition it demonstrated the presence of 16 amino acid long C-terminal sequence found in some but not all α subunits (Fig. 1B). Deletion analysis showed that the presence of both C-terminal as well as N-terminal amino acids sequences was required for PR-39 binding (Fig. 1C). In order to confirm the PR39- α 7 subunit interaction in-vivo, anti-PR39 antibody was used to immunoprecipitate PR39 protein from ECV-PR39, ECV-E4 and mock-transfected ECV cells. Western blotting of the immunoprecipitate from ECV-PR39 and ECV-E4 but not wild type ECV cells with anti- α 7 subunit antibody demonstrated the presence of a 29kDa band corresponding to the known size of α 7 subunit protein (Fig. 1D). The evidence therefore reveals that PR39 peptide interacts with α 7 subunit of proteasome in ECV cells.

Experiment 2:

To test the ability of PR-39 peptide to affect proteasome function in-vivo, the effect of PR-39 peptide administration on I κ B α processing was assessed. The results are illustrated by Figs. 2A-2D respectively.

1 Fig. 2A shows a Western analysis of I κ B α expression in ECV cells. The results
2 show that pretreatment of cultured ECV cells with lactacystin (10 μ M, 4th lane) or stable
3 expression of full length (ECV-PR39) or PR39 exon 4 (ECV-E4) constructs inhibited
4 TNF- α -induced degradation of I κ B.

5 Thus, tumor necrosis factor (TNF)- α induces rapid degradation of I κ B α - a
6 function that is blocked by the proteasome inhibitor lactacystin. However, Western
7 analysis of I κ B α levels after TNF- α treatment demonstrated comparable levels of I κ B α
8 expression in both ECV-PR39 and ECV-E4 cells to that seen in ECV cells pre-treated
9 with lactacystin.

10 Fig. 2B shows the effect of PR39, MG132 and lactacystin pretreatment on I κ B α
11 expression in U937 cells following TNF- α treatment. Note similar extent of inhibition of
12 I κ B α degradation by TNF- α following pretreatment with PR39, MG132 or lactacystin.
13 Thus, it is clear that pretreatment of U937 cells with PR39 blocked TNF- α induced I κ B α
14 degradation in a manner that was similar to the degree of inhibition seen with MG132 and
15 lactacystin.

16 Fig. 2C demonstrates the reversibility of PR39 inhibition of proteasome activity.
17 U937 cells were pretreated with PR39, MG132 or lactacystin for 45 min. After that time,
18 the cells were extensively washed with fresh medium. 45 min later TNF- α (1 ng/ml) was
19 added to the medium and the extent of I κ B α degradation was determined 10 min later by
20 Western blotting. Note preservation of I κ B α in lactacystin-treated cells but not PR39-
21 treated cells. Thus, unlike lactacystin but similar to MG132, PR-39 peptide mediated
22 inhibition of I κ B α degradation was rapidly reversible.

Finally, to show that PR-39 inhibition of I κ B α degradation affected NF κ B-dependent transcription, ECV cells were transiently transfected with a NF κ B-Luc reporter construct containing a tandem of four NF κ B binding sites in front of luciferase cDNA. The results of Fig. 2D show that stimulation with TNF- α induced a significant increase in luciferase activity that was completely inhibited by pretreatment with PR39.

Accordingly, the true functional significance of PR39-mediated inhibition of I κ B α degradation in ECV cells transiently transfected with pNF κ B-Luc reporter vector (Clontech) is clearly shown by Fig. 2D. Pre-treatment with PR39 completely inhibited TNF- α -induced increase in luciferase activity. *p<0.01 vs. control (Luc activity in the absence of TNF- α).

Experiment 3:

To demonstrate directly the ability of PR-39 peptide to inhibit proteasome-mediated protein degradation, preparations of eukaryotic 20S proteasomes were tested for their ability to induce proteolysis of various synthetic peptides in-vitro. The results are graphically illustrated by Figs. 3A-3D respectively.

For determination of proteasome activity, 5 μ l of 1:10 diluted rabbit muscle 20S proteasome preparation (courtesy of Dr. M. Sherman, BBRI, Boston) was incubated at room temperature in an assay buffer (20 mM Tris-HCl pH 8.0, 0.5 mM EDTA and 0.01% SDS) with 20 μ M of four different proteasome substrates (CalBiochem, 539140-3) and PR39 or other proteasome inhibitor at indicated concentration. The extent of substrate degradation was monitored continuously by fluorescence spectrophotometry (380 nm excitation, 460 nm emission Hitachi F-2000) for 10 min.

1 Figs. 3A-3D reveal that the PR-39 peptide inhibited, in a dose-dependent manner,
2 degradation of all 4 peptides tested. PR-39 peptide was as potent as lactacystin or MG132
3 in inhibiting degradation in three of the four peptides tested and was considerably more
4 potent in inhibiting degradation of the Z-Leu-Leu-Glu-AMC peptide.

5

6 Experiment 4:

7 To test the effect of PR39 treatment on cellular levels of other proteasome-
8 dependent proteins, the in-vivo expression of p105 and p50 NF κ B, HSP70 and HIF-1 α
9 within transfected and wild type ECV cells was determined. The results are illustrated by
10 Figs. 4A-4C respectively.

11 Fig. 4A shows the results of a Western blot analysis of HIF-1 α , p50 and p105
12 NF κ B expression in wild type ECV cells and ECV-E4 and ECV-PR39 clones. It is noted
13 that an increase in HIF-1 α expression occurs (but not p105 or p50 NF κ B expression) in
14 ECV-E4 and ECV-PR 39 clones. Thus, while there was a significant increase in
15 expression of HIF-1 α and I κ B in PR39 transfected or treated compared to wild type cells,
16 there was no significant change in expression of either HSP70 or NF κ B - demonstrating
17 that effects of PR39-proteasome interaction are selective.

18 Since increased expression of HIF-1 α is known to result in increased transcription
19 of a number of angiogenesis-related molecules including VEGF, Northern analysis of
20 VEGF mRNA levels in wild type and PR39 transfected ECV cells was performed. The
21 results are shown by Fig. 4B. As expected, there was a significant increase in expression
22 of both of these genes in ECV-PR39 and ECV-E4 cells compared to ECV controls.

Finally, exposure to proteasome inhibitor lactacystin is known to induce rapid cell death. To test the effect of PR39 on cell survival, growth rates of ECV cells treated with PR39 peptide were assessed. The results are shown by Fig. 4C. 50,000 ECV cells were cultured in 10% FBS-M199 in the absence (control) or presence of 10 μ M of PR39, lactacystin or MG132. Note that while exposure to PR39 did not affect cell growth, exposure to lactacystin or MG132 substantially inhibited cell growth. Thus, following 3 days of PR39 exposure, treated cells demonstrated normal growth compared to controls while those cells exposed to lactacystin demonstrated markedly reduced survival.

Experiment 5:

To demonstrate the stimulation of angiogenesis directly in living cells and tissues via the introduction of PR-39 peptide, a mice matrigel assay system was employed. Growth factor-depleted Matrigel pellets containing 5 μ g of PR39, 50 ng of FGF2 or saline (control) were inserted intraperitoneally into C57BL/6 mice. Ten days later the pellets were removed, sectioned and stained with anti-CD31 antibody. The number of vessels was determined in multiple sections using a digital camera and Optimas 5.0 software. The results are shown by Figs. 5A-5C and Fig. 6 respectively.

Figs. 5A-5C are representative sections from control, PR39 and FGF2 impregnated Matrigel pellets and Fig. 6 provides a quantitative analysis of vascularity. Clearly, the results of the representative sections and the graphic quantitative evaluations demonstrate that insertion of growth-factor depleted Matrigel pellet containing PR-39 peptide induced intense vessel growth that exceeded that seen with implantation of pellets containing 50 ng/ml of bFGF.

Experiment 6:

To demonstrate the efficiency of shorter-length peptides which collectively are members of the PR-39 derived oligopeptide family in stimulating angiogenesis in-vivo, a novel peptide, PR11, composed of the first 11 amino acid residues [N-terminal end] of the native PR-39 sequence was purposely synthesized. The amino acid sequence of PR11 is as follows:

1 2 3 4 5 6 7 8 9 10 11
Arg-Arg-Arg-Pro-Arg-Pro-Pro-Tyr-Leu-Pro-Arg

To introduce the short-length PR11 peptide in-vivo, a mouse Matrigel assay system was utilized. In sum, either 5 µg/ml of PR11 peptide or 5 µg/ml of native PR-39 peptide were individually placed into a growth factor-depleted Matrigel pellet; and then each prepared Matrigel pellet was inserted into the peritoneal cavity of a mouse. After 14 days intraperitoneal placement, each pellet was removed from its living host; and each pellet was examined for evidence of new vascularity. The results are graphically presented by Fig. 7. Note that the bar graph of Fig. 7 shows the number of blood vessels [mean±SD] per 10 high power fields (HPF).

As evidenced by Fig. 7, the analysis of Matrigel pellet vascularity after 14 days incubation in-vivo demonstrated significant induction of angiogenesis in both the PR11 and the native PR-39 pellets. The control Matrigel pellets, however, showed no evidence of angiogenesis as such. Clearly therefore, the short-length PR11 peptide is fully efficacious and effective in stimulating angiogenesis in-vivo.

Conclusions:

(1) The described experiments and empirical data have demonstrated that PR-39 peptide has the ability to selectively alter activity of 20S proteasome in human endothelial cells by interacting with proteasomes in a reversible manner. This interaction leads to suppression of I κ B and HIF-1 α degradation while not affecting expression of other proteasome-dependent proteins such as p105 NF κ B or HSP70. Unlike other proteasome inhibitors, treatment with PR39 is not associated with any cellular cytotoxicity. Thus, PR39 and its related peptides provide a unique and unforeseen means of regulating cellular function and stimulating angiogenesis.

(2) Several observations also set PR39 apart from the conventionally known proteasome inhibitors. First, PR39-mediated inhibition of I κ B α degradation is demonstrably reversible, unlike that of lactacystin. Second, long-term exposure of several cell types to PR39 did not result in any cytotoxicity, in contrast to the rapid cell death typically observed following cell treatment with lactacystin or MG132. This observation shows that PR39 peptide differentially affects processing of various and different intracellular proteins. Also supporting this view is the observation that while increasing HIF-1 α expression, PR39 administration had no meaningful effect on the expression of either NF κ B or HSP70. Third, PR-39 peptide modulation of proteasome activity plays a functional role since the observed increased expression of HIF-1 α was directly associated with an increased expression of its target genes, VEGF and *flt-1*.

1 The present invention is not to be limited in form nor restricted in scope except by
2 the claims appended hereto.

- 48 -

What we claim is:

1. A method for stimulating angiogenesis within a targeted collection of viable cells in-situ, said method comprising the steps of:

identifying a collection of cells comprising viable cells in-situ as a target for stimulation of angiogenesis;

providing means for effecting an introduction of at least one member selected from the group consisting of the PR-39 oligopeptide collective to the cytoplasm of said targeted collection of cells;

introducing at least one member of the PR-39 oligopeptide collective to the cytoplasm of said targeted collection of cells using said effecting means;

allowing said introduced PR-39 oligopeptide collective member to interact with such proteasomes as are present within the cytoplasm of said targeted collection of cells whereby

(a) at least some of the proteasomes interact with said PR-39 oligopeptide collective member, and

(b) at least a part of the proteolytic activity mediated by said interacting proteasomes becomes selectively altered, and

(c) the selectively altered proteolytic activity of said interacting proteasomes results in a stimulation of angiogenesis in-situ within the targeted collection of viable cells.

2. A method for selective inhibition of proteasome-mediated degradation of peptides in-situ within a collection of viable cells, said method comprising the steps of:

- identifying a collection of cells comprising viable cells in-situ as a target;
- providing means for effecting an introduction of at least one member selected from the group consisting of the PR-39 oligopeptide collective to the cytoplasm of said targeted collection of cells;
- introducing at least one member of the PR-39 oligopeptide collective to the cytoplasm of said targeted collection of cells using said effecting means;
- allowing said introduced PR-39 oligopeptide collective member to interact with such proteasomes as are present within the cytoplasm of said targeted collection of cells whereby
 - (a) at least some of the proteasomes interact with the PR-39 oligopeptide collective member, and
 - (b) at least a part of the proteolytic activity mediated by said interacting proteasomes becomes markedly altered, and
 - (c) the markedly altered proteolytic activity of said interacting proteasomes results in a selective inhibition of proteasome-mediated degradation of peptides in-situ within the targeted collection of cells.

3. The method as recited in claim 1 or 2 wherein said collection of viable cells includes at least one type of cell selected from the group consisting of endothelial cells,

myocytes and myoblasts, fibrocytes and fibroblasts, epithelial cells, osteocytes and osteoblasts, neuronal cells and glial cells, erythrocytes, leukocytes, and progenitor cells of all types.

4. The method as recited in claim 1 or 2 wherein said collection of cells comprises at least one tissue selected from the group consisting of myocardium, skeletal muscle, smooth muscle, an artery, a vein, lung, brain, kidney, spleen, liver, gastrointestinal tissue, nerve tissue, limbs, and extremities.
5. The method as recited in claim 1 or 2 wherein the means for an introduction of a PR-39 oligopeptide collective member include one selected from the group consisting of catheter-based introduction means, injection-based introduction means, infusion-based introduction means, localized intravascular introduction means, liposome-based introduction means, receptor-specific peptide introduction means, slow releasing means for peptide secretion in living cells and sequestered organisms.
6. The method as recited in claim 1 or 2 wherein the means for an introduction of a PR-39 oligopeptide collective member includes the DNA sequences coding for PR-39 oligopeptides of different sizes inserted in a suitable vector for transfection and subsequent expression of peptides within said cells.
7. The method as recited in claim 1 or 2 wherein said method is practiced under *in-vivo* conditions.

8. The method as recited in claim 1 or 2 wherein said method is practiced under in-vitro conditions.

9. The method as recited in claim 1 or 2 wherein degradation of I κ B α is selectively inhibited.

10. The method as recited in claim 1 or 2 wherein degradation of HIF-1 α is selectively inhibited.

11. A family of PR-39 derived oligopeptides whose members individually cause a selective inhibition of proteasome-mediated degradation of peptides in-situ after introduction intracellularly to a viable cell, each member of said oligopeptide family being:

- a peptide less than 39 amino acid residues in length;
- at least partially homologous with the N-terminal amino acid residue sequence of the native PR-39 peptide;
- able to interact in-situ with such proteasomes as are present within the cytoplasm of the cell; and
- able to alter markedly the proteolytic activity of said interacting proteasomes such that a selective increased expression of specific peptides occurs in-situ.

12. The PR-39 derived oligopeptide family as recited in claim 11 whose membership includes a peptide comprised of 15 amino acid residues whose sequence is Arg-Arg-Arg-Pro-Arg-Pro-Pro-Tyr-Leu-Pro-Arg-Pro-Arg-Pro-Pro.

13. The PR-39 derived oligopeptide family as recited in claim 11 whose membership includes a peptide comprised of 11 amino acid residues whose sequence is Arg-Arg-Arg-Pro-Arg-Pro-Pro-Tyr-Leu-Pro-Arg.

14. The PR-39 derived oligopeptide family as recited in claim 11 whose membership includes a peptide comprised of 8 amino acid residues whose sequence is Arg-Arg-Arg-Pro-Arg-Pro-Pro-Tyr.

ABSTRACT OF THE INVENTION

The present invention provides both a method and means for regulating angiogenesis within living cells, tissues, and organs in-situ. The regulation is performed using native PR-39 peptide or one of its shorter-length homolog, for interaction with such proteasomes as one present in the cytoplasm of viable cells. The result of PR-39 peptide interaction with proteasomes is a decrease in the intracellular degradation of active peptides such as HIF-1 α and a consequential stimulation of angiogenesis in-situ.

Fig. 1A

		α ₇ subunit	Growth/LacZ+
Mouse	MSSIGTYDLSASTFSPDGR	VFQVEYAMKA	VENSTAIGI RCKDGVVFGV
human	-----	-----	-----
EKIVLSKLYE	EGSNKRLENV	DRHVGMAVAG	LLADARSLAD IAREEASNR
SNFGYNIPLK	HLADRVMAMYV	HAYTLYSAVR	PFGCSFMGLS YSNNDGAOLY
MIDPSGVSYG	YWGCAGKAR	QAKTEIEKL	QMKEEMTCRDV VKEVAKIYI
VHDEVKDKAF	ELELSWVGEI	TKGRHEIVPK	DIREEAKYA KESIKEEDES
DDDNM	-----	-N-----	-I-----

Fig. 1C

	α ₇ subunit	Growth/LacZ+	
	1	255	+++
	210	255	-
	172	255	-
	171	1	-

Fig. 1D

ECV ECV-E4 ECV-PR₃₉ ECV homogenate



Fig. 1B

	C terminal tails	Net Charge	
α1	AERD	-1	α7
α2	A	0	
α3	KKHEEEAKAEREKKEKEQKEKDK	+1	
α4	EKEKEENEKKQQKKAS	+2	
α5	EEPRQPKAQPADEPAKADEPMEH	0	
α6	-----	-3	
α7	AKESLKEEDESDDDNM	-6	

Fig. 2A

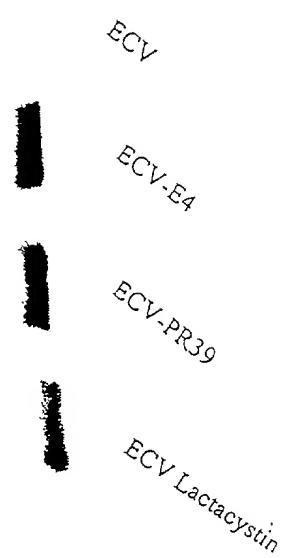


Fig. 2B

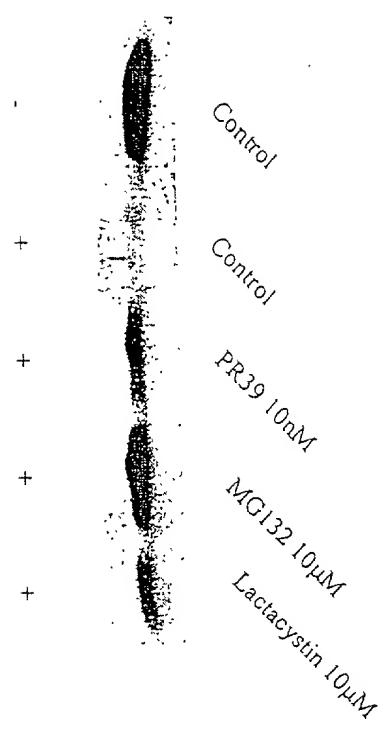


Fig. 2C

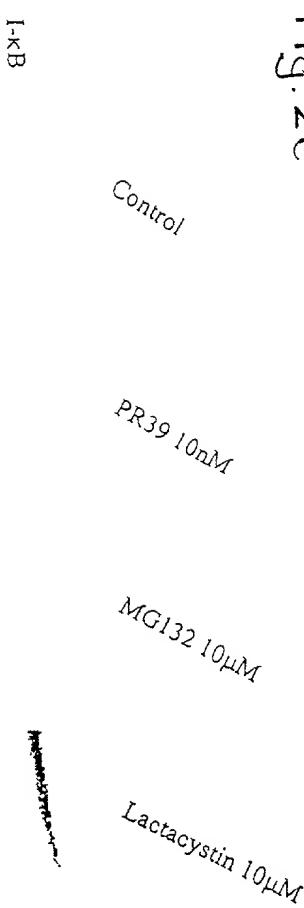
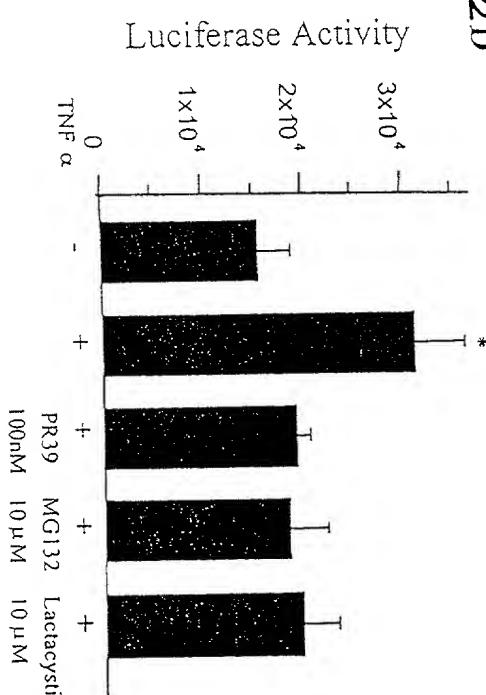


Fig. 2D



TNF-α

Fig. 3 A

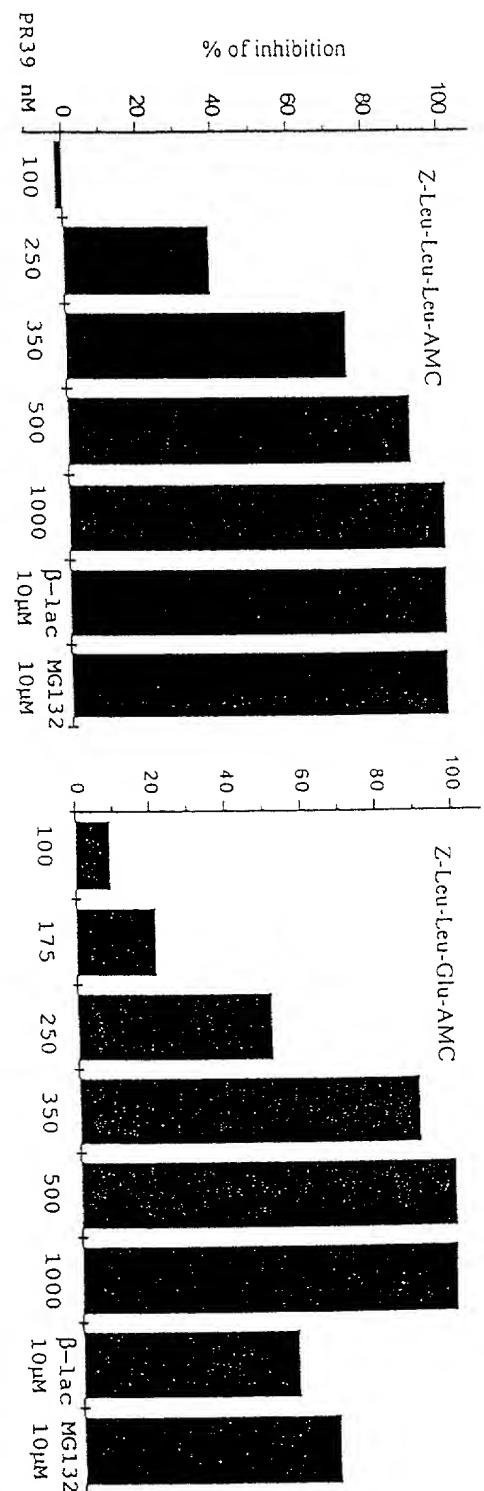


Fig. 3 C



Fig. 3 B

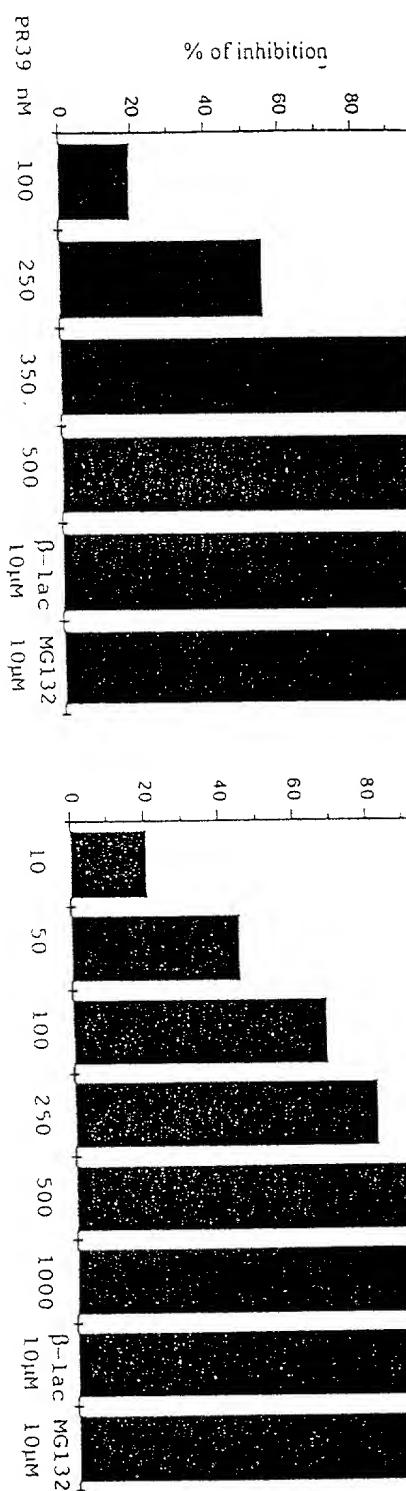


Fig. 3 D

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Fig. 4A

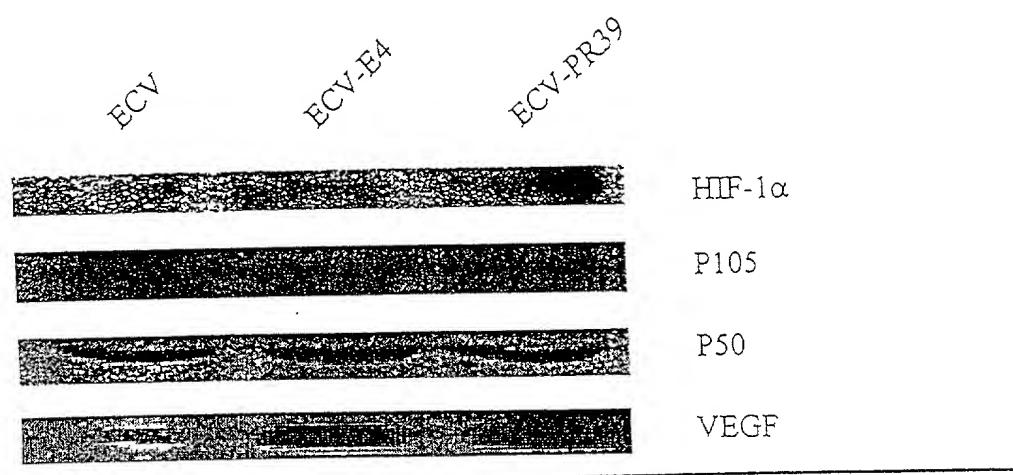
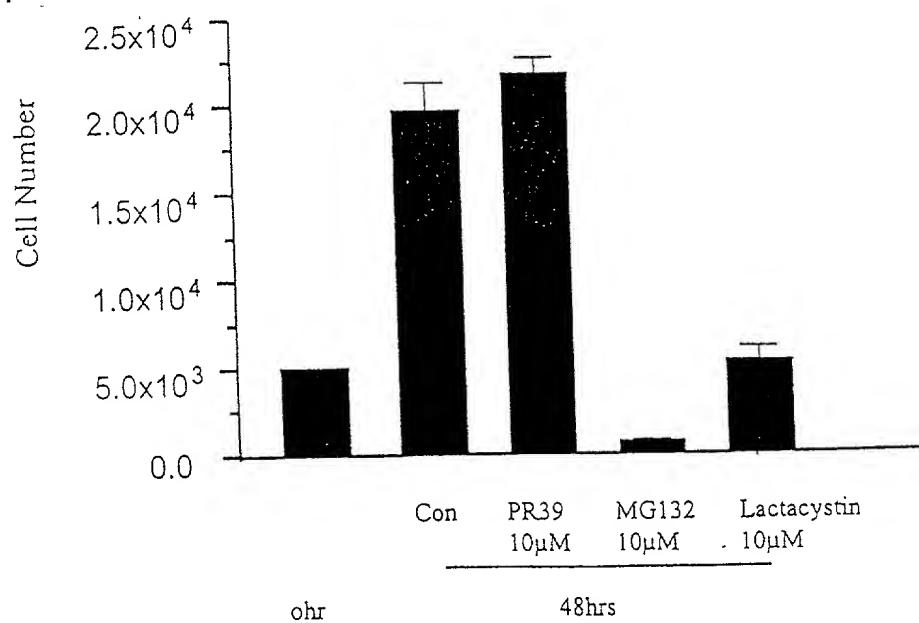


Fig. 4B



Fig. 4C



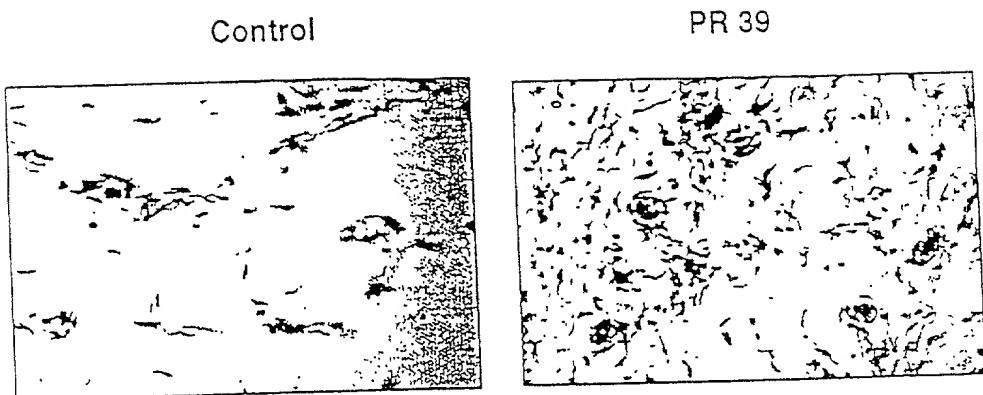


Fig. 5 A

Fig. 5 B

Fig. 5 C

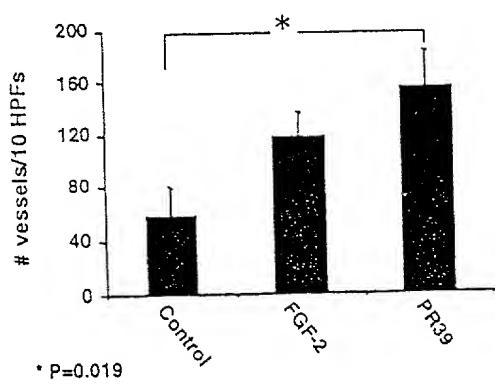


Fig. 6

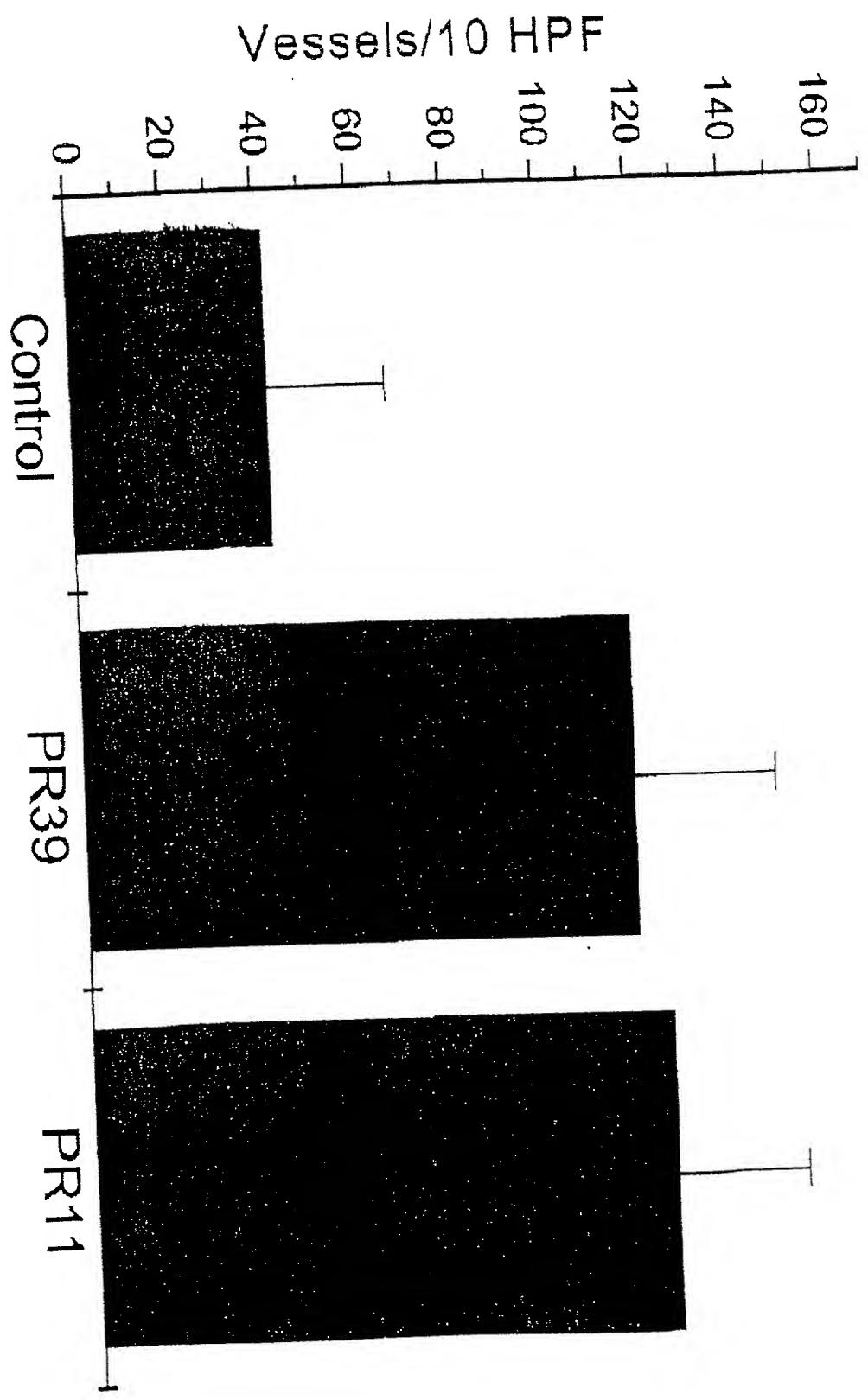


Fig. 7

Attorney's Docket No. BIS-043/CIP

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled "METHOD FOR PR-39 REGULATED STIMULATION OF ANGIOGENESIS", the specification of which:

XXXX is attached hereto; or
was filed on _____ as United States
Application Serial No. _____; or
was filed on _____ as PCT International
Application No. _____ and was amended on _____.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 35 U.S.C. 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119 (a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below any foreign application for patent or inventor's certificate, or any PCT international application having a filing date before that of the application on which priority is claimed:

PRIOR FOREIGN APPLICATION(S)

Priority claimed

..... NONE
(Number) (Country) (Day/month/year/filed) Yes No

I hereby claim the benefit under 35 U.S.C. 119(e) of any United States Provisional Patent Application(s) listed below.

..... NONE

XXXX I hereby claim the benefit under 35 U.S.C. 120 of any United States application(s), or 365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application(s) in the manner provided by the first paragraph of 35 U.S.C. 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 C.F.R. 156 which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application:

U.S. Patent Application Serial No. 09/276,868 filed March 26, 1999, now pending.

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney to prosecute this application and transact all business in the Patent and Trademark Office connected therewith; and, in addition, to act as Agent on my behalf before the competent International Authorities and before the National Authorities for any designated countries in connection with any and all international applications filed or to be filed by the undersigned.

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

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